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A *Campylobacter jejuni znuA* Orthologue Is Essential for Growth in Low-Zinc Environments and Chick Colonization▽

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Campylobacter jejuni **infection is a leading cause of bacterial gastroenteritis in the United States and is acquired primarily through the ingestion of contaminated poultry products. Here, we describe the *C. jejuni* orthologue of ZnuA in other gram-negative bacteria. ZnuA (Cj0143c) is the periplasmic component of a putative zinc ABC transport system and is encoded on a zinc-dependent operon with Cj0142c and Cj0141c, which encode the other two likely components of the transport system of *C. jejuni*. Transcription of these genes is zinc dependent. A mutant lacking Cj0143c is growth deficient in zinc-limiting media, as well as in the chick gastrointestinal tract. The protein is glycosylated at asparagine 28, but this modification is dispensable for zinc-limited growth and chick colonization. Affinity-purified FLAG-tagged Cj0143c binds zinc in vitro. Based on our findings and on its homology to *E. coli* ZnuA, we conclude that Cj0143c encodes the *C. jejuni* orthologue of ZnuA.**

Campylobacter jejuni infection is a leading cause of bacterial gastroenteritis worldwide. Symptoms of acute campylobacteriosis include self-limiting watery to bloody diarrhea with nausea, fever, and abdominal cramping. *C. jejuni* is a microaerophilic gram-negative bacterium that commonly colonizes the gastrointestinal tract of broiler chickens. Passage of *C. jejuni* to humans occurs primarily through consumption of contaminated poultry products (38).

An important trait of bacterial pathogens is their ability to take up trace elements. Iron is commonly considered the most important trace element necessary for bacterial growth inside of mammalian hosts. However, other trace divalent metals play critical roles during infection, including zinc and manganese (11, 42). Zinc plays both structural and catalytic roles in over 300 proteins within *Escherichia coli*, because it is incorporated into regulatory proteins, ribosomal proteins, and many other enzymes. Although zinc is essential, excess zinc is toxic to cells because it competes with other metals for enzyme binding sites, and internal zinc homeostasis is tightly regulated in both eukaryotic and bacterial cells. The intracellular concentration of zinc within *E. coli* is 0.2 mM; however, little or no free zinc exists within the cytoplasm (30).

Several transport systems have been identified that are involved in intracellular zinc homeostasis. Under conditions of limited metal availability, such as those within the host, a high-affinity zinc transport system is responsible for the uptake of zinc, as initially reported for *E. coli* (30). This system is encoded by the *znuABC* genes and is part of the family of ATP-binding cassette (ABC) transporters. ZnuA is a periplasmic metallochaperone, ZnuB is a membrane permease, and ZnuC is an ATPase component (30, 31).

The *znuABC* genes in *E. coli* are regulated by Zur (*z*inc-*u*ptake *r*egulator), a member of the Fur (*f*erric-*u*ptake *r*egulator) family of metalloregulatory proteins. In the presence of zinc, Zur forms a dimer and binds a palindromic sequence, termed the Zur box, thereby repressing expression of the *znuABC* genes. Zur is sensitive to femtomolar concentrations of zinc within *E. coli*, once again demonstrating the stringent regulation of zinc within cells (28).

The amount of zinc available within a host is assumed to be limited, similar to that of iron, and the ZnuABC transporters are required for virulence by several pathogens, including *E. coli*, *Salmonella enterica*, *Brucella abortus*, *Neisseria gonorrhoeae*, *Pasteurella multocida*, and *Haemophilus* species (20, 25, 30, 31). *C. jejuni* must survive in zinc-deprived environments during its infectious cycle; hence, it is likely that zinc homeostasis plays a major role in its ability to survive within hosts as well.

To assess the role zinc plays in *C. jejuni* growth and within a host, we identified and characterized ZnuA in *C. jejuni*. Our results demonstrate that Cj0143c encodes the metallochaperone ZnuA. ZnuA is essential for *C. jejuni* growth in zinc-limiting media, as well as within the chick gastrointestinal tract. ZnuA is N glycosylated by the Pgl system, but we demonstrate that glycosylation of the protein does not affect its function (36).

MATERIALS AND METHODS

Strains and plasmids. *Campylobacter jejuni* strain 81-176 was isolated from a human clinical case of campylobacteriosis. DRH212 is a streptomycin-resistant derivative of *C. jejuni* 81-176 (13). The plasmid pBW210 is derived from pEco101 (3) and contains a C-terminal Flag sequence under the control of a chloramphenicol acetyltransferase (*cat*) promoter (3, 19, 38). All strains and plasmids used in this study are shown in Table 1.

Bacterial growth conditions. *C. jejuni* was grown at 37°C in a tri-gas incubator containing 85% N_2, 10% O_2, and 5% CO_2 on plates with Mueller-Hinton (MH) medium containing 10 μg/ml trimethoprim (TMP). To obtain growth curves, bacteria were grown overnight on solid MH medium containing 10 μg/ml TMP, collected, and resuspended to an optical density at 600 nm (OD_{600}) of 0.4. For 1 ml of medium, 4 μl of a 1:10 dilution of this suspension was added. Cultures

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli strains		
DH5α		10
JM101		New England Biolabs
DH5α/pRK212.1	Contains conjugative plasmid for conjugation of plasmid DNA into *Campylobacter*	5
C. jejuni strains		
81-176	Clinical isolate	21
DRH212	81-176 *rpsL*(Sm)	13
TK429	DRH212 ΔCj0143c	19
TK430	Cj0143c3NQ	This study
TK211	DRH212 Δ*pglB*; nonmotile variant	19
Plasmids		
pGEM-T	Cloning vector system	Promega
pECO101	pRY108 derivative with *cat* promoter in XhoI-BamHI site; Kn^r	3
pBW210	pECO101 derivative with N-terminal Flag in frame with BamHI site; Kn^r	This study
pLD36	pBW120 with Cj0143c coding sequence cloned into BamHI/XhoI site	This study
pLD37	pBW210 with Cj0143c3NQ coding sequence cloned into BamHI/XhoI	This study

[a] *rpsL*(Sm), *rpsL* gene with resistance to streptomycin.

were grown statically under atmospheric conditions described above. MH broth was utilized for growth curves unless otherwise stated; *Campylobacter* defined medium (CDM) was also utilized to grow *C. jejuni* when stated as such (24).

When necessary, media were supplemented with antibiotics at the following concentrations: chloramphenicol, 15 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; TMP, 10 μg ml^{-1}; streptomycin, 2 mg ml^{-1}. All *C. jejuni* strains were stored in MH broth with 20% glycerol at −80°C.

E. coli strains were grown in Luria-Bertani (LB) broth or agar. When necessary, medium was supplemented with antibiotics at the following concentrations: kanamycin, 50 μg ml^{-1}; chloramphenicol, 30 μg ml^{-1}. All *E. coli* strains were stored at −80°C in LB broth with 20% glycerol.

Construction of DRH212 ΔCj0143c. A chromosomal, in-frame deletion of Cj0143c was constructed in a streptomycin-resistant derivative of *C. jejuni* 81-176 (DRH212) by using the method described by Hendrixson et al. (13). The Cj0143c gene was amplified by PCR using primers 500 bp upstream (5′-CTTTAGTGT TGGTATGCAAG-3′) and downstream (5′-CTGCTAAGAATCTTCCAGCA-3′) of the coding sequence, with primers based on the genomic sequence of *C. jejuni* NCTC 11168. The product was cloned into a pGEM-T vector. The resulting plasmid (pTK007) was then digested with PflMI and treated with T4 DNA polymerase to make blunt ends. A *cat-rpsL* cassette was then ligated into the gene and transformed into DH5α, resulting in the plasmid pTK059. pTK059 was electroporated into DRH212, constructing Cj0143c::*cat-rpsL* (TK106).

The deletion of Cj0143c was made by *Pfu* DNA polymerase-mediated mutagenesis using Cj0143c on pTK007 to make pTK259, using the primers 5′-TT AGGGATATTTTATACTTTCACACAAGCTACTGCTGATGCTTTTCTCA TAATTTATAA-3′ and 5′-TTATAAATTATGAGAAAAGGCATCAGCAGT AGCTTGTGTGAAAGTATAAAATATCCCTAA-3′. This suicide plasmid was electroporated into the *cat-rpsL*–inactivated mutant TK106. Transformants were selected on medium with 2 mg of streptomycin ml^{-1} and screened for the loss of the *cat-rpsL* cassette by plating on MH agar with 10 μg chloramphenicol ml^{-1}. Deletion was verified by PCR and sequencing.

Construction of the DRH212 Cj0143c3NQ strain. In order to study how glycosylation affects Cj0143c, we performed site-directed mutagenesis. Three putative sequons were initially identified in the coding sequence of Cj0143c at residues N28, N216, and N169, based on the initial definition of the sequon (N-X-S/T) (27). An allele of Cj0143c was constructed in which all three of these sites were replaced with glutamines. Introduction of point mutations in Cj0143c was performed by site-directed mutagenesis using *Pfu* DNA polymerase as described above. The primer pairs 5′-GCTAAAAATTTAGAGCAAGAACAAC AAACTAGCAGCAATTTAGTTAGTG-3′ and 5′-CACTAACTAAATTGCT GCTAGTTTGTTGTTCTTGCTCTAAATTTTTAGC-3′, 5′-CTTGGACATAT TTTGCAAAACGCTATCAACTTACGCAAATTCCTGTAT-3′ and 5′-CTAC AGGAATTTGCGTAAGTTGATAGCGTTTTGCAAAATATGTCCAAG-3′, and 5′-GAAAAAGTTTTTACGGATAAATTTAAACAACAATTTTCAAAA CAACAAGTTGTAAATATGC-3′ and 5′-GCATATTTACAACTTGAAGTTT TGAAAATTGTTGTTTAAATTATCCGTAAAAACTTTTTC-3′ were used to alter residues N28, N216, and N169, respectively. This construct was then used in allelic replacement to alter Cj0143c on the chromosome, creating the Cj0143c3NQ strain. Subsequent to our mutagenesis experiment, the sequon was redefined by Kowarik et al. as D/E-X-N-Y-S/T,X,Y ≠ P (22). Based on this definition, N28 is the sole glycosylation sequon in the *C. jejuni* Cj0143c strain (Fig. 1A).

Both the ΔCj0143c and the Cj0143c3NQ strains grew like the wild type in MH medium over time, suggesting that the mutations contained in these strains did not induce a growth defect in rich media (data not shown). Although the Cj0143c3NQ mutant contains extraneous mutations, because the mutations do not interrupt the function of the protein (see Results), we used the triple-mutation strain in our studies.

Construction of Flag-tagged proteins. The coding sequences from the second codon up to, but not including, the last codon of Cj0143c and that of Cj0143c3NQ were amplified by PCR. These products were cloned into the BamHI site of pBW210. The plasmids were introduced into *E. coli* DH5α/pRK212.1 and conjugated into the DRH212, ΔCj0143c, Δ*pglB*, and Cj0143c3NQ strains.

Bacterial fractionation. *C. jejuni* was grown under microaerobic conditions at 37°C for 24 h on MH agar containing 10 μg/ml TMP. Bacteria were resuspended in MH broth and centrifuged at 10,000 × *g* for 10 min at 4°C. The pellet was resuspended in 10% (wt/vol) sucrose and 30 mM Tris-HCl (pH 7.4). One millimolar EDTA was added, and the suspension was stirred for 10 min at room temperature. The mixture was centrifuged at 10,000 × *g* for 10 min at 4°C. The pellet was resuspended in cold 0.5 mM $MgCl_2$ and stirred on ice for 10 min. This mixture was then centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was used as the periplasmic fraction. The pellet was resuspended in 10 mM HEPES (pH 7.3) and frozen at −80°C for 1 h. The suspension was sonicated six times for 10 s each time or until the lysate cleared. To remove cell debris, the suspension was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was centrifuged at 100,000 × *g* for 1 h. The pellet of this suspension was used as the membrane (insoluble) fraction, and the supernatant was used as the cytoplasmic (soluble) fraction. Protein content was measured with a Bio-Rad protein assay.

Immunoblotting with anti-FLAG antibody. Western blots were performed as previously described (19). Briefly, *C. jejuni* strains expressing pEco101-FLAG were resuspended to an OD_{600} of 0.8. One ml of the suspension was centrifuged and resuspended in 100 μl of 2× loading buffer. The samples were boiled for 10 min and subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins samples were transferred to nitrocellulose paper and blocked with 5% milk in Tris-buffered saline (TBS) plus Tween (20 mM Tris 7.4-150 mM NaCl-0.1% Tween 20).

Zinc binding assay. Strains expressing FLAG-tagged Cj0143c and FLAG-tagged Cj0143c3NQ were grown for 18 h on MH agar plates containing 10 μg ml^{-1} TMP. Periplasmic fractions were collected from the two strains. EZ-view Red anti-FLAG M2 affinity gel beads (Sigma) were washed with TBS (50 mM Tris HCl, 150 mM NaCl [pH 7.4]) according to the protocol supplied by the manufacturer. The bacterial periplasmic lysates were mixed with the anti-FLAG

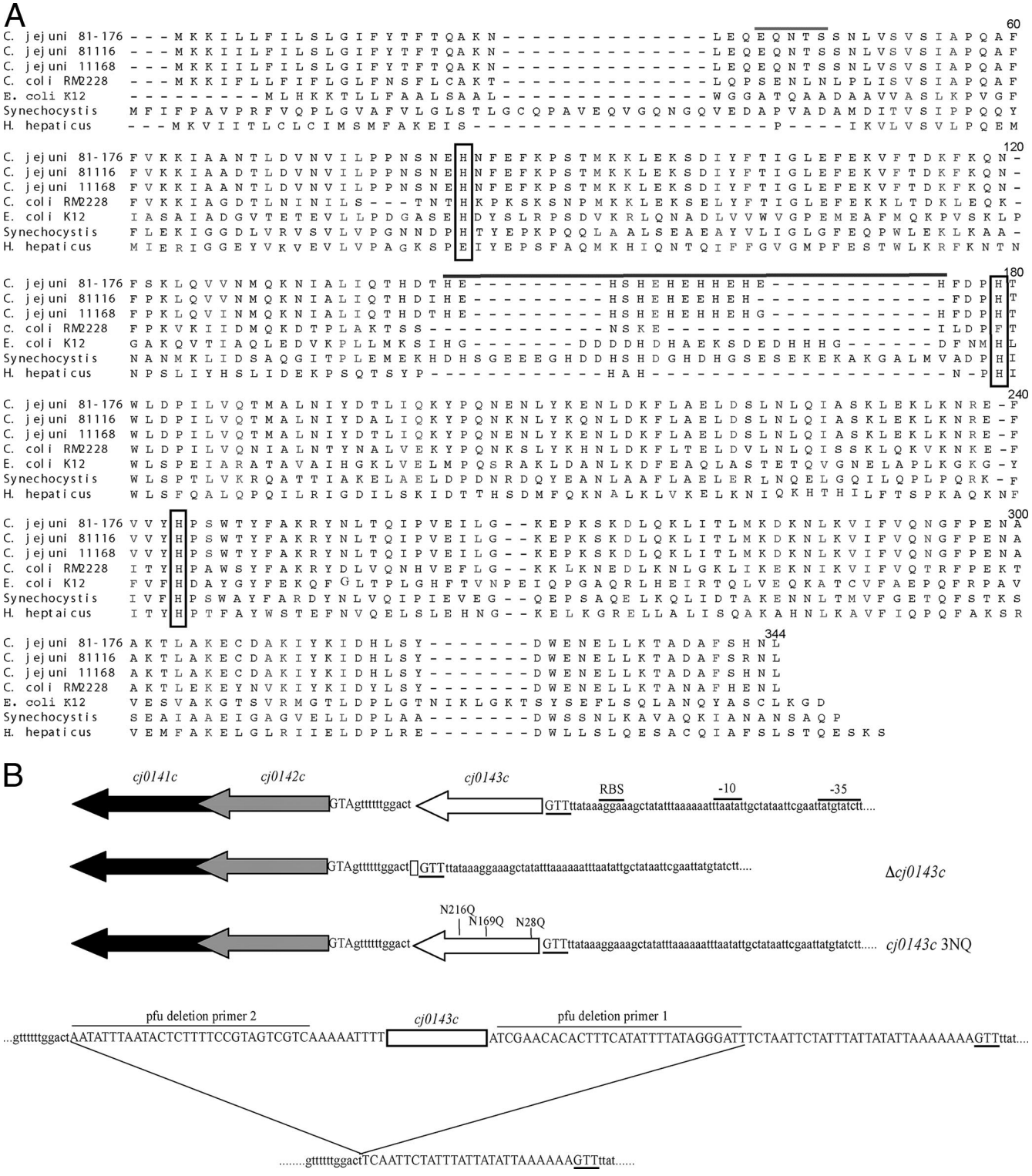

FIG. 1. (A) ClustalW alignment of the ZnuA sequences from *Campylobacter jejuni* 81-176, *C. jejuni* 11168, *C. jejuni* 81116, *Campylobacter coli* RM2228, *E. coli* K-12, *Synechocystis* 6803, and *Helicobacter hepaticus* 51449. Conserved residues and similar charged residues demonstrate a high degree of homology, especially around the zinc binding pocket. The conserved zinc-binding histidines are denoted by black boxes. A hypervariable region which contains a predicted zinc-binding arm in *Synechocystis* is conserved between the *C. jejuni* strains (indicated by a long horizontal line above residues 142 to 175). The sequon or the site recognized for N glycosylation by the Pgl system in *C. jejuni* is indicated by a short horizontal line above residues 43 to 47. (B) Genetic organization of Cj0141c, Cj0142c, and Cj0143c with consensus promoter sequences (black lines above sequence) and ribosome binding site (RBS) upstream of Cj0143c. An in-frame deletion of Cj0143c was constructed, as well as an allele containing point mutations at three putative N glycosylation sites. A schematic of the deletion of Cj0143c is shown with lowercase letters representing a noncoding sequence and uppercase letters denoting a coding sequence. The TTG start site is underlined for Cj0143c.

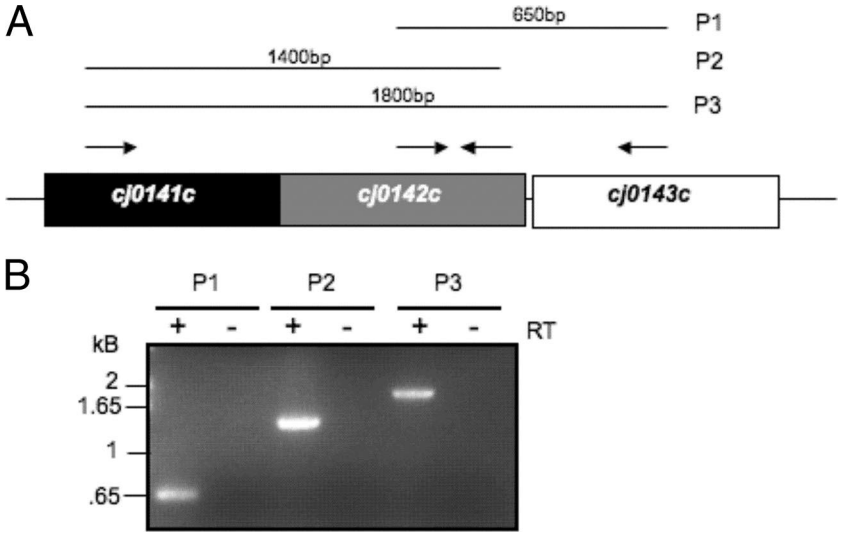

FIG. 2. (A) RT-PCR was performed with the loci to determine whether Cj0141c, Cj0142c, and Cj0143c are cotranscribed. The three primer sets (P1, P2, and P3) were designed to span the three genes. Their predicted product sizes (bp) are reported. (B) RT-PCR with RNA extracted from wild-type *C. jejuni* DRH212 using the primer sets shown in panel A. Results for the RT (+) and no-RT control (−) are represented for each primer set.

M2 affinity gel beads and incubated overnight at 4°C on a rocking shaker. The beads were washed four times with TBS. The bead-protein complex was incubated with HBS (50 mM HEPES-KOH [pH 7.5], 0.2 M NaCl) buffer only, with 1 mM $ZnSO_4$, or with 1 mM EDTA for 1 h at 4°C on a rocking shaker. The samples were washed four times, and the protein was eluted from the beads by incubation with 0.1 M glycine HCl (pH 3.5) for 5 min at room temperature. Beads were centrifuged at 8,000 × *g* for 10 s. The supernatant was collected and neutralized with 0.5 M Tris HCl (pH 7.4), 1.5 M NaCl. The sample was divided in half, and total protein was determined by using a Bio-Rad protein assay. The metal content of the sample was determined by using the 4-(2-pyridylazo)resorcinol (PAR) assay. A portion of the samples was analyzed by SDS-PAGE and stained with Coomassie blue to monitor protein purification.

PAR assay. The PAR assay (18) is used to quantify the amount of zinc within a sample. Briefly, after purification, the protein samples were digested with 50 μg/ml proteinase K in HBS for 30 min at 56°C. An equal volume of HBS with 0.2 mM PAR was added to the digested sample and read with a plate reader at 490 nm. The quantity of zinc was determined by comparison to a standard curve of $ZnSO_4$ and was normalized to the amount of protein in each sample, as determined by a Bio-Rad assay following purification.

Chick colonization assay. The chick colonization assay was performed as previously described (14). Briefly, day-old chicks were orally inoculated with 10^4 CFU of *C. jejuni* diluted in phosphate-buffered saline. After 7 days, chicks were euthanized, and their ceca were removed. Cecal contents were weighed, diluted, and plated onto MH agar containing 10 μg ml^{-1} TMP, 30 μg ml^{-1} cefoperazone, and 2 mg ml^{-1} streptomycin. *C. jejuni* colonies were counted, and CFU g^{-1} cecal matter were reported.

RESULTS

Characterization of Cj0143c. As part of an effort to characterize glycoproteins of *C. jejuni* (20), the putative glycoprotein Cj0143c was identified in *C. jejuni* as having extensive homology to ZnuA in *E. coli*, a high-affinity zinc binding protein for an ABC transporter (30). A ClustalW alignment of Cj0143c sequences from *C. jejuni* 81-176, other *C. jejuni* strains, *Campylobacter coli*, *Helicobacter hepaticus*, *Synechocystis* spp., and *E. coli* was performed (Fig. 1A). *C. jejuni* 81-176 *znuA* had high homology with the *znuA* gene from other *C. jejuni* strains (96 to 99%) but limited homology to the *znuA* genes from *C. coli* RM228 (61%), *E. coli* (21.5%), *Synechocystis* (32.8%), and *H. hepaticus* (26%). The three conserved histidine residues in *E. coli* ZnuA comprise a high-affinity binding site (Fig. 1A). Beginning at residue 162 in the *C. jejuni* 81-176 sequence is a 14-amino-acid sequence, of which 13 residues are His (H) or Glu (E). This H/E-rich domain per se is found in the other *C. jejuni* strains; however, orthologues from other species have a His/Asp-rich region (Fig. 1A). *Synechocystis* ZnuA contains a 20-amino-acid sequence rich in histidine and acidic residues that forms a loop, as determined by crystal structure (1). This loop in the *Synechocystis* ZnuA protein is not essential for zinc binding to the high-affinity binding site of ZnuA but may play a regulatory role in transport (37).

The Cj0143c gene was first identified as encoding a glycoprotein, modified by the N glycosylation system in *C. jejuni* (40). The Pgl system assembles a conserved heptasaccharide and transfers it onto periplasmic proteins by the transferase PglB (36). PglB recognizes a conserved sequence, termed the sequon, D/E-X-N-Y-S/T,X,Y ≠ P (22). A sequence matching this consensus, EQNTS (Fig. 1A), is conserved in all *Campylobacter jejuni* strains.

Based on homology, we predict that Cj0143c (*C. jejuni* 81-176 gene number CJJ81176_0179) and its two adjacent open reading frames (ORFs), Cj0141c (CJJ81176_0177) and Cj0142c (CJJ81176_0178), together form a zinc ABC transporter similar to ZnuABC (see Fig. 1B).

Cj0141c, Cj0142c, and Cj0143c are cotranscribed, and expression is elevated in low-zinc media. To determine whether Cj0141c, Cj0142c, and Cj0143c form an operon, reverse transcription (RT)-PCR was performed with RNA extracted from wild-type *C. jejuni* grown in MH broth containing 10 μM EDTA, using three primer sets spanning the putative operon (Fig. 2A). A transcript encoding all three ORFs would be predicted to be amplified by primer pair P3. A 650-bp product was detected for P1, a 1,400-bp product for P2, and an 1,850-bp product for P3 in the RT reactions (Fig. 2B). These species are indicative of transcription across all three ORFs. Based on

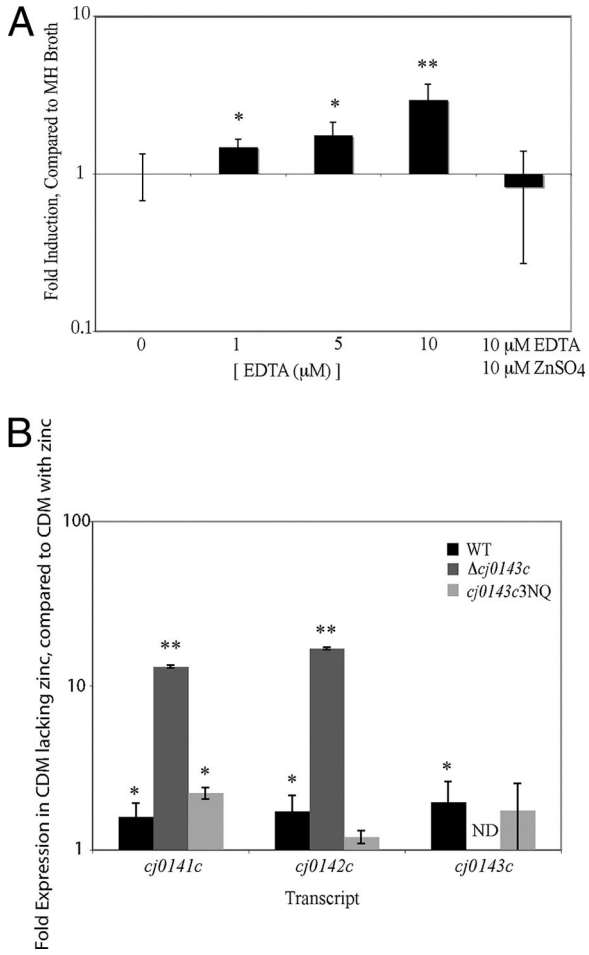

FIG. 3. (A) Quantitative RT-PCR of Cj0143c transcript levels in wild-type *C. jejuni* grown under the indicated conditions: MH broth, 1 μM EDTA, 2 μM EDTA, and 10 μM EDTA and 10 μM EDTA plus 10 μM $ZnSO_4$. Compared to MH broth transcript levels, Cj0143c is expressed more in the presence of EDTA. *, $P < 0.05$; **, $P < 0.005$. (B) Quantitative RT-PCR of Cj0141c, Cj0142c, and Cj0143c transcript levels in wild-type *C. jejuni* (black), ΔCj0143c (dark gray), and cj0143c 3NQ (light gray) strains grown in CDM lacking zinc are compared to those in bacteria grown in MH medium. Transcription of Cj0143c in ΔCj0143c was not detected, while Cj0141c and Cj0142c transcripts were detected, demonstrating a nonpolar ΔCj0143c mutation. **, $P < 0.005$.

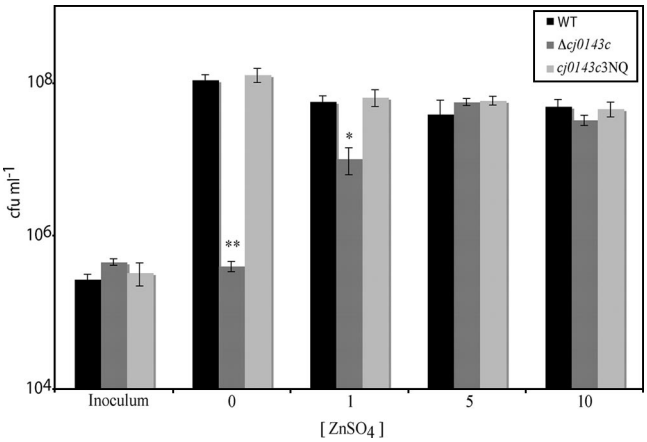

FIG. 4. Growth of wild-type (WT), ΔCj0143c, and Cj0143c3NQ strains at 24 h in defined medium without the addition of zinc. ΔCj0143c is unable to grow in the defined medium lacking zinc. When the medium was supplemented with increasing concentrations of $ZnSO_4$, the growth of ΔCj0143c was restored to wild-type levels. *, $P < 0.05$; **, $P < 0.005$.

these results, we conclude that Cj0143c, Cj0142c, and Cj0141c are cotranscribed.

To assess whether expression of the operon is influenced by zinc concentrations, quantitative RT-PCR was performed with the Cj0143c transcript from cells grown in the presence of various concentrations of EDTA, a common chelator of divalent cations. Wild-type *C. jejuni* was cultured for 24 h in MH broth, in MH broth with 1, 2, and 10 μM EDTA, and in 10 μM EDTA with 10 μM Zn. The increases in Cj0143c transcript levels under different conditions were compared to transcript levels in bacteria grown in MH medium (Fig. 3A). Expression of Cj0143c was two- to threefold increased in the presence of EDTA (Fig. 3A). When zinc was added to EDTA, the transcription level resembled that of growth in MH medium, indicating that the expression of Cj0143c in the presence of EDTA is zinc dependent.

Deletion of Cj0143c results in a growth defect in zinc-limiting media. We hypothesized that Cj0143c is required for *C. jejuni* growth in zinc-limiting environments. We constructed a strain with an in-frame chromosomal deletion of Cj0143c (ΔCj0143c), as well one expressing an allele with altered glycosylation sites (Cj0143c3NQ), as described in Materials and Methods. Quantitative RT-PCR with primers for Cj0141c, Cj0142c, and Cj0143c transcripts was performed with wild-type and ΔCj0143c and Cj0143c3NQ strains grown in CDM containing or lacking zinc (Fig. 3B). Cj0143c transcript was not detected in ΔCj0143c, while both Cj0141c and Cj0142c transcripts were detected, confirming that the in-frame deletion mutation of Cj0143c is not polar on downstream transcription. Furthermore, in a comparison of expression during growth with and without added zinc, Cj0141c and Cj0142c were expressed at 10-fold-higher levels in the ΔCj0143c mutant than in the wild-type and Cj0143c3NQ strains (Fig. 3B). We think this occurs because the ΔCj0143c mutation creates a zinc-starved condition within the bacteria. Although the medium does not contain added zinc, contaminating trace amounts of zinc may be present.

The growth of the ΔCj0143c and Cj0143c3NQ strains was similar to that of the wild type in rich MH broth cultures. However, the growth of ΔCj0143c was severely attenuated after 24 h when cultured in CDM lacking the added zinc (Fig. 4). Growth of the ΔCj0143c strain was restored when $ZnSO_4$ was added to CDM (Fig. 4). Culturing these strains under conditions that lacked other individual trace elements, including Fe^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, and Mn^{2+}, was also tested, and ΔCj0143c growth was not significantly attenuated under any other condition, suggesting that the requirement for the ΔCj0143c strain is specific for zinc-limited growth (data not shown).

We also tested zinc-limited growth by using EDTA, hypothesizing that the ΔCj0143c strain would be challenged under

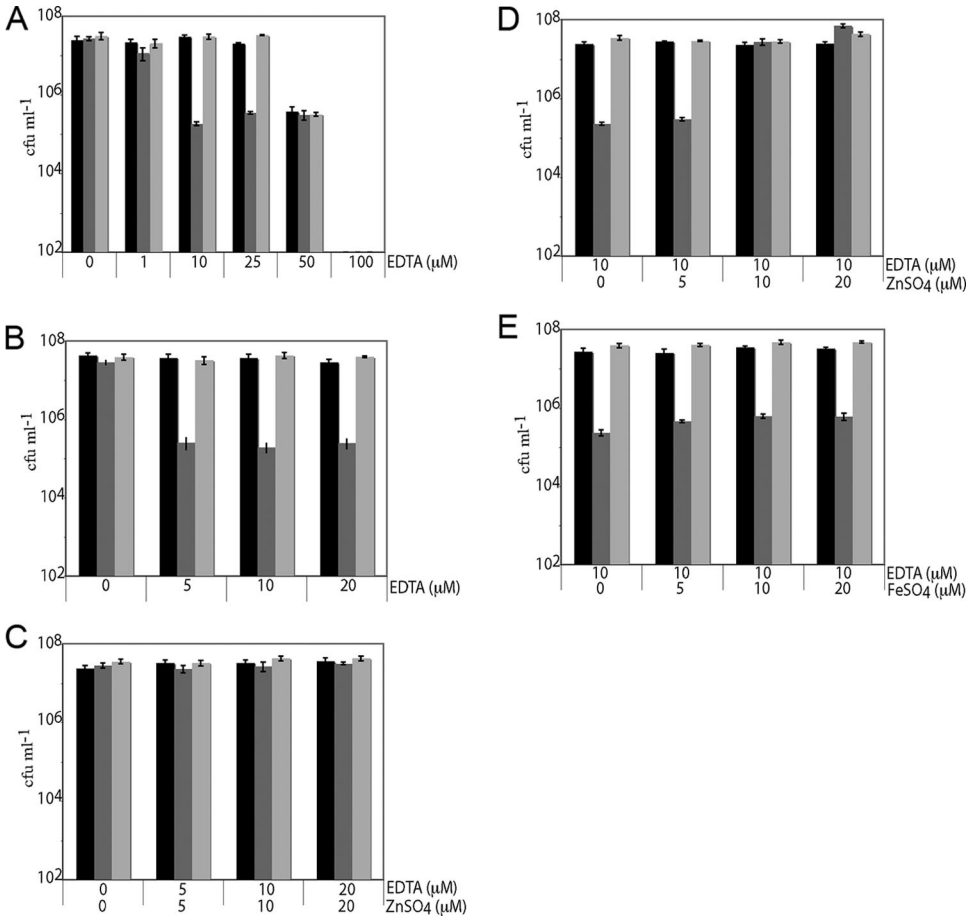

FIG. 5. (A to C) Wild-type (black), ΔCj0143c (dark gray), and Cj0143c3NQ (light gray) strains grown for 24 h in MH broth containing increasing levels of EDTA. (C) Strains were grown in MH broth containing increasing levels of EDTA and supplemented with increasing amounts of $ZnSO_4$, demonstrating that $ZnSO_4$ is able to rescue the growth defect of ΔCj0143c in MH broth containing high levels of EDTA. (D to E) Increasing amounts of either $ZnSO_4$ (D) or $FeSO_4$ (E) were added to MH broth containing 10 μM EDTA. Growth of ΔCj0143c is more restricted in the presence of EDTA and was rescued only with $ZnSO_4$. These data demonstrate that Cj0143c is required for growth in low-zinc media. **, $P < 0.005$.

this zinc-depleted condition. We grew wild-type, ΔCj0143c, and Cj0143c3NQ strains in MH broth with increasing concentrations of EDTA for 24 h, with an inoculum of 10^5 CFU ml^{-1}. At 10 and 25 μM EDTA, the wild-type and the Cj0143c3NQ mutant strains grew well but the ΔCj0143c mutant did not (Fig. 5A). All three strains were unrecoverable in the presence of 100 μM EDTA (Fig. 5A). This coincides with the toxicity of EDTA for *Helicobacter pylori*, with a MIC as low as 0.1 mM EDTA (26). Addition of equimolar concentrations of $ZnSO_4$ to MH broth containing EDTA rescued the growth of the ΔCj0143c strain (Fig. 5B); in contrast, addition of $FeSO_4$ at concentrations of up to 20 μM was unable to rescue growth of the ΔCj0143c strain in 10 μM EDTA (Fig. 5C).

Taken together, these data suggest that Cj0143c is necessary for *C. jejuni* growth in low-zinc environments and that it is specific for zinc.

Cj0143c is glycosylated at a single site. To assess the glycosylation state of Cj0143c, we constructed plasmids expressing Cj0143c and Cj0143c3NQ with C-terminal FLAG epitopes (pCj0143c-FLAG and pCj0143c3NQ-FLAG, respectively) from plasmid pBW210. These constructs were introduced into the wild-type, ΔCj0143c, and Δ*pglB C. jejuni* backgrounds. A *C. jejuni* Δ*pglB* mutant lacks the glycosyl transferase *pglB*, which is necessary for the final step in N-linked protein glycosylation (20, 36). All strains grew similarly to the wild type in rich and in low-zinc media, suggesting that the FLAG-tagged proteins remain functional and complement the ΔCj0143c mutant (data not shown).

Immunoblots of whole-cell lysates of the indicated strains grown on MH agar for 24 h were performed and probed with anti-FLAG antibody. The wild-type and the ΔCj0143c strain carrying pCj0143c-FLAG expressed two species recognized by the anti-FLAG antibody (Fig. 6A). A single species was observed for the wild type and the ΔCj0143c strain carrying pCj0143c3NQ-FLAG. When pCj0143c-FLAG was expressed in the Δ*pglB* mutant of *C. jejuni*, which is unable to carry out N-linked protein glycosylation, only a single species was detected with anti-FLAG antibody. This species has a mobility similar to that of the Cj0143c3NQ strain and to that of the lower of the two species observed in cells expressing pCj0143c-FLAG. Given these results and the updated sequon consensus sequence, we conclude that Cj0143c

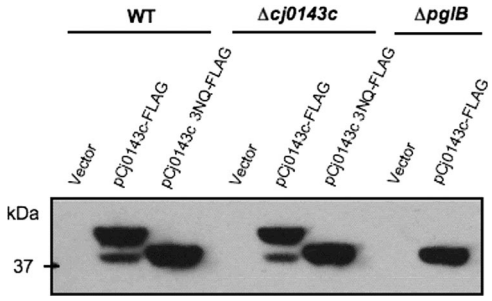

FIG. 6. Glycosylation states of Cj0143c. Whole-cell lysates were collected from wild-type (WT) DRH212 and ΔCj0143c strains and from the Δ*pglB* strain containing vector only, pCj0143c-FLAG, or pCj0143c3NQ-FLAG. Samples were normalized based on OD_{600}, run on SDS-PAGE gels, and blotted with anti-FLAG antibody.

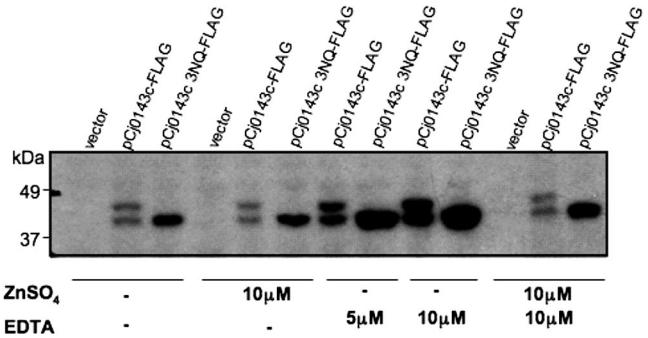

FIG. 8. Cj0143c-FLAG stabilizes with increasing amounts of EDTA. Strain ΔCj0143c carrying pCj0143c-FLAG or pCj0143c3NQ-FLAG and strain ΔCj1496c carrying pCj1496c-FLAG were grown for 24 h in MH broth, MH plus 5 μM EDTA, MH plus 10 μM EDTA, and MH plus 10 μM EDTA and 10 μM $ZnSO_4$. Cells were normalized by OD_{600}. Cj0143c-FLAG and Cj0143c3NQ-FLAG were more abundant in the presence of EDTA.

is glycosylated at a single site, most likely at residue N28. We conclude that the upper band shown in Fig. 6B is the glycosylated species and the lower band is the unglycosylated species.

Cj0143c localization. Based on its homology to ZnuA, we predicted that Cj0143c is localized to the periplasm. To test this, subcellular fractions of cells expressing pCj0143c-FLAG were analyzed with anti-FLAG antibody. Bands corresponding to the glycosylated and unglycosylated forms of Cj0143c in wild-type, ΔCj0143c, and Δ*pglB* backgrounds were located in the periplasmic fraction only (Fig. 7). The activity level of isocitrate dehydrogenase, a cytoplasmic enzyme, was used to demonstrate the purity of each fraction. Isocitrate dehydrogenase activity was highest in the cytoplasmic fraction (Fig. 7), and activity in the periplasmic fraction might have reflected contamination. However, little or no signal was detected for Cj0143c-FLAG in the cytoplasmic or the membrane fraction, suggesting that Cj0143c is localized to the periplasm.

Cj0143c is more stable in the presence of EDTA. Due to the fact that intracellular concentrations must be strictly controlled, zinc must be maintained at strict levels. External environmental zinc levels may change rapidly in the various hosts and under conditions that *C. jejuni* encounters. *C. jejuni* must respond quickly to these changes, and therefore, it is reasonable to expect that this system could be regulated at both the transcriptional and the protein levels.

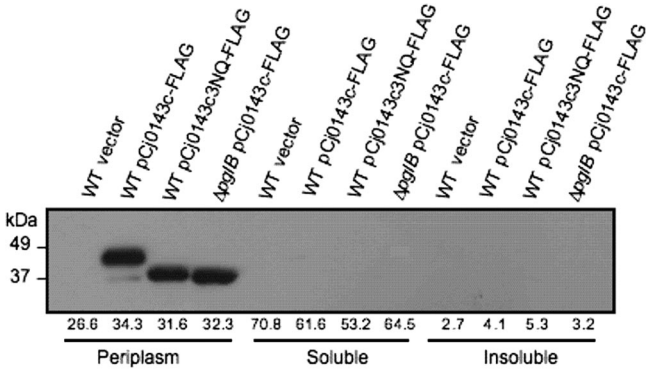

FIG. 7. Localization of Cj0143c-FLAG. Wild-type (WT) DRH212 expressing vector alone, pCj0143c-FLAG, or pCj0143c3NQ-FLAG was fractionated into periplasmic, soluble, and insoluble fractions. The samples were run on SDS-PAGE gels and blotted with anti-FLAG antibody. Cj0143c-FLAG and Cj0143c3NQ-FLAG are localized to the periplasmic fraction in wild-type and Δ*pglB* backgrounds. The blot shown is representative of three separate localizations. Isocitrate dehydrogenase activity levels for the samples are shown below the lanes.

We asked whether either the protein levels or the glycosylation of Cj0143c was affected based on various growth conditions such as temperature (37°C versus 42°C) or growth phase or various levels of zinc. We grew the ΔCj0143c strain carrying pCj0143c-FLAG under a variety of conditions, including increasing levels of EDTA (Fig. 8). The quantities of Cj0143c-FLAG and Cj0143c3NQ-FLAG in the ΔCj0143c strain increased with the addition of EDTA (Fig. 8). In this experiment, Cj0143c-FLAG was expressed from the Cat^r promoter on plasmid pEco101, so any increase in the amount of Cj0143c-FLAG under zinc-limiting conditions is not due to transcriptional regulation.

Cj1496c is a previously characterized periplasmic glycoprotein in *C. jejuni* (20). Cj1496c-FLAG was used as a control to demonstrate that the EDTA effect was specific for Cj0143c-FLAG (data not shown) and not due to the global effect that EDTA could have on *C. jejuni*, such as through the activity of a metal-dependent protease or plasmid-specific stabilization.

Cj0143c binds zinc in vitro. To test the zinc-binding capacity of Cj0143c, PAR was used in an in vitro zinc binding assay. We took advantage of the fact that a PAR-Zn^{2+} complex forms a color that can be measured at 490 nm (18). The proteins Cj0143c-FLAG, Cj0143c3NQ-FLAG, and Cj1496c-FLAG were purified using anti-FLAG affinity beads as described in Materials and Methods. Cj1496c-FLAG was used as a control in this assay, as it does not contain a zinc-binding site.

The purified proteins were incubated with EDTA or $ZnSO_4$ for 1 h. Proteins were eluted from the beads, and the zinc content of each sample was measured by adding 0.2 mM PAR. The amount of zinc (μM) was determined by comparing the absorbance to that of a standard curve of PAR/$ZnSO_4$. The samples were standardized to the quantity of purified protein in each sample.

We determined the concentration of zinc per μg of purified protein for vector alone, for Cj0143c-FLAG, for Cj0143c3NQ-

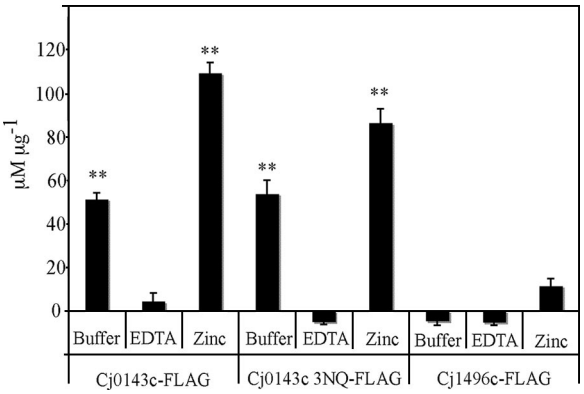

FIG. 9. Cj0143c and Cj0143c3NQ bind zinc in vitro. Cj0143c-FLAG and Cj0143c3NQ-FLAG were immobilized on Sepharose beads conjugated to anti-FLAG antibody. The protein-bead complex was incubated with buffer, EDTA, or $ZnSO_4$. Protein quantity (μg) was determined by Bio-Rad assay. The amount of zinc was quantified by measuring the absorbance of the PAR-zinc complex in each sample and calculating the total μM concentration of zinc. The values are μM of zinc per μg of purified protein. Cj0143c and Cj0143c3NQ-FLAG are able to bind zinc in vitro and at similar levels. Cj1496c-FLAG, a periplasmic-localized glycoprotein, is not able to bind zinc in vitro. **, $P < 0.005$.

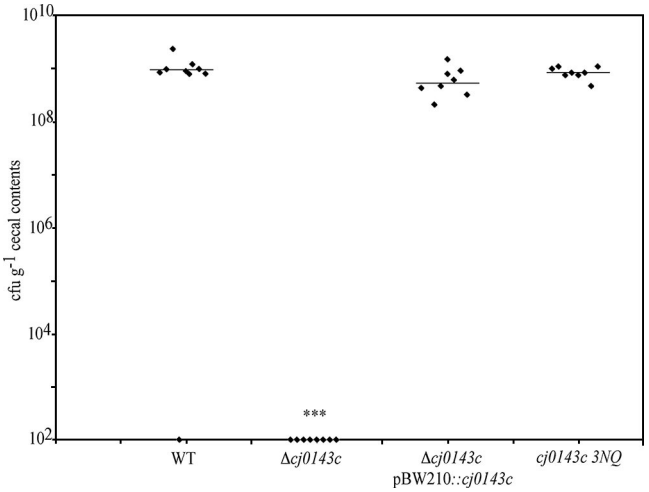

FIG. 10. Cj0143c, but not the glycosylation of Cj0143c, is required for colonization of chick ceca. Day-old chicks were inoculated with 10^4 CFU of wild type, ΔCj0143c, ΔCj0143c containing pCj0143c-FLAG, or Cj0143c3NQ. After 7 days, the chicks were euthanized, and their cecal contents were plated for *C. jejuni* CFU/g of cecal matter, with a limit of detection of 10^2 CFU/ml. Each point represents an individual chick. ***, $P < 0.001$.

FLAG, and for Cj1496c-FLAG incubated with buffer, 1 mM EDTA, or 1 mM $ZnSO_4$ (Fig. 9). Zinc was detected in the samples incubated with buffer alone for Cj0143c-FLAG and for Cj0143c3NQ-FLAG at concentrations of 50 μM Zn^{2+} per μg of protein. This most likely represents zinc that remained bound to the proteins during purification. Addition of 1 mM EDTA reduced the detection of zinc in the protein complex considerably. With the addition of 1 mM $ZnSO_4$, both Cj0143c-FLAG and Cj0143c3NQ-FLAG contained approximately 100 μM Zn^{2+} per μg of protein. The samples of Cj1496c-FLAG bound zinc poorly or below detection levels.

These results demonstrate that Cj0143c-FLAG and Cj0143c3NQ-FLAG are able to bind zinc in vitro. The increase in zinc content with the addition of $ZnSO_4$ most likely represents the saturation of binding sites, including the predicted high-affinity zinc-binding site (Fig. 1A), as well as the zinc binding arm that we speculate exists in the protein (Fig. 1A).

Cj0143c is required for chick colonization. *Campylobacter jejuni* colonizes the chick gastrointestinal tract to high levels. Factors that influence this natural colonization are beginning to be uncovered (11) and may serve as targets for developing approaches to limit chick colonization and thereby reduce human exposure. To assess whether Cj0143c is necessary for *C. jejuni* interactions with the host, the wild-type *C. jejuni* and ΔCj0143c strains, the ΔCj0143c strain containing pCj0143c-FLAG, and the Cj0143c3NQ strain were orally inoculated into day-old White Leghorn chicks at a dose of 10^4 CFU. After 7 days, the cecal contents of the infected chicks were plated at CFU per gram of cecal contents. Both the Cj0143c3NQ mutant and the ΔCj0143c strain containing pCj0143c-FLAG colonized chicks at wild-type levels; however, ΔCj0143c was unrecoverable at our limit of detection, signifying that Cj0143c is necessary for colonization (Fig. 10).

DISCUSSION

Campylobacter jejuni likely encounters various levels of trace elements, including zinc, during its life cycle. Zinc is an essential trace element but can also be toxic to bacteria, and therefore, intracellular concentrations must be tightly regulated (30). Bacteria regulate zinc homeostasis through zinc uptake and efflux systems.

A number of bacterial zinc transport systems have been characterized, including ZntA, ZupT, and ZnuABC. The ZntA system is a conserved bacterial zinc efflux system. ZupT is involved in uptake of zinc in *E. coli* and is a member of the ZIP family of zinc transporters (9). Based on sequence homology and previous characterization, *C. jejuni* contains the ZntA (Cj1155c) and ZupT (Cj0263) systems. Here, we propose that Cj0143c, Cj0141c, and Cj0142c encode the proteins of the ZnuABC zinc uptake system, with Cj0143c as the zinc binding protein (ZnuA), Cj0142c as the ATPase component (ZnuC), and Cj0141c as the integral membrane protein (ZnuB).

The ZnuABC system is essential for a number of bacterial pathogens in their colonization of and virulence in hosts (6, 17, 21, 26, 27). This work shows that *znuABC* in *C. jejuni* is essential for growth in low-zinc environments, as well as for colonization of chick ceca. The necessity of *C. jejuni* ZnuA in the colonization of chick ceca, along with previous literature showing that *znuA* is an important factor for survival of *E. coli*, *Brucella abortus*, *Haemophilus* spp., *Neisseria gonorrhoeae*, and *Pasteurella multocida* in hosts, suggest that available zinc is scarce within mammalian hosts (8, 20, 25, 30, 31). This mechanism is similar to that of the low availability of iron, for which there are specific host binding proteins, transferrin and lactoferrin (4). The intestines are the major site of zinc absorption, where intestinal metallothionein is thought to sequester zinc in the intestinal walls (2). Metallothionein is an intracellular, cysteine-rich protein with a high binding affinity for heavy-

metal ions, especially zinc. It is involved in zinc homeostasis as well as in providing zinc for metalloenzymes involved in defense against pathogens (2, 33). Whether metallothionein plays a direct role in the defense against microbial pathogens is currently unknown. However, mice deficient in metallothionein are more sensitive than wild-type mice to *Helicobacter pylori* gastritis (33). Furthermore, serum and plasma zinc concentrations are depressed when chickens are infected with *Salmonella enterica* serovar Gallinarum or *E. coli*, suggesting a potential antimicrobial strategy of limiting the availability of zinc (15, 32, 34). Competition for trace elements such as iron and zinc between a host and pathogen is critical for establishing infection. As chickens are one of the main sources for acquiring *C. jejuni*-related illness, factors that significantly decrease the ability of *C. jejuni* to colonize this food source could be a target for drug development.

Because it is repressed by Zur, the ZnuABC system is highly regulated in *E. coli*. Zur is part of the family of Fur proteins. Fur family proteins regulate metal homeostasis and act as global regulators of growth and, in many cases, of virulence (12, 17). Zur represses zinc-responsive genes in the presence of zinc and is known to be sensitive to femtomolar quantities of zinc in *E. coli* (28). Based on BLAST analysis, *C. jejuni* does not contain a *zur* homolog.

Fur regulates zinc-responsive genes within *Pasteurella multocida* (8), in the absence of Zur. The sequence upstream of Cj0143c does not contain a Zur or Fur box, as previously defined (Fig. 1B) (29). Previous microarray analysis demonstrated that Cj0142c was 2.2-fold upregulated in response to iron-limited conditions but not in a *fur* mutant (16). In a different microarray screen, Cj0141c and Cj0143c were not regulated in response to iron availability (29). Based on these data and sequence analysis, we predict that the *C. jejuni znuABC* genes are not regulated by Fur. We demonstrated by RT-PCR that the *znuABC* genes in *C. jejuni* 81-176 are coexpressed under zinc-limiting conditions. In the absence of regulation by Zur and Fur, we suggest that another form of regulation may be occurring for this system in *C. jejuni*.

PerR (peroxide stress regulator) was previously identified as a Fur homolog in *C. jejuni* that is responsive to iron limitation, as well as to peroxide stress (35). In *Bacillus subtilis*, PerR is a peroxide-sensitive regulator that binds Fe^{2+} or Mn^{2+} as a cofactor and represses genes including *katA* (catalase), *ahpPCF* (alkyl hydroperoxide), *mrgA* (Dps-like DNA binding protein), *hemAXCDBL* (heme biosynthesis operon), *zosA* (zinc-uptake system), *fur*, and *perR* (6). Gaballa and Helmann further demonstrated that in *B. subtilis*, zinc uptake by ZosA is regulated by PerR and contributes to oxidative stress resistance (7). Along with data showing Fur regulation of zinc-responsive genes in *Pasteurella multocida*, these data demonstrate the ability for cross-talk of cation-responsive gene regulation within bacterial systems. In the absence of a *zur* homolog in *C. jejuni*, alternatives such as cross-regulation by PerR or Fur or an as-yet uncharacterized system are attractive explanations for the transcriptional regulation of the *znuABC* system.

ZnuA from *C. jejuni* binds zinc in vitro, irrespective of glycosylation, as we demonstrated using the PAR assay. Approximately 50 μM zinc per μg of protein was detected for Cj0143c-FLAG when incubated with buffer alone, and 100 μM zinc per μg protein was detected with the addition of 1 mM $ZnSO_4$. The quantity 50 μM of zinc per μg of protein equates to 1.78 atoms of zinc for every molecule of ZnuA. This is approximately what was reported for *E. coli* ZnuA, which contains a high-affinity binding site and a lower-affinity site. (39). The addition of 1 mM $ZnSO_4$ presumably saturated the protein with zinc with approximately 100 μM zinc per μg of protein. This equates to 3.56 atoms of zinc per molecule of ZnuA, demonstrating the ability of *C. jejuni* ZnuA to bind to multiple zinc molecules.

ZnuA in *Synechocystis* contains a flexible loop of histidine and acidic residues, which has been hypothesized to perform a chaperone or regulatory function (11, 40). At very low concentrations of zinc, only the high-affinity site binds zinc. When zinc concentrations are high, zinc association with the flexible loop may block zinc transport through structural changes (11, 33). *C. jejuni* ZnuA has a region containing histidine and glutamates (Fig. 1A) and, similar to *Synechocystis* ZnuA, can bind multiple zinc molecules in high zinc concentrations (Fig. 9). Furthermore, our data suggest that Cj0143c-FLAG is more stable under zinc-limiting conditions. We propose that zinc binding to the histidine-rich arm of Cj0143c results in a less stable conformation of the protein, leading to a second level of regulation. This may represent a previously uncharacterized system of cation regulation. Future work in the flexible loop region of ZnuA, possible regulators such as PerR, and protein stability will provide insight into how this system is tightly regulated in *C. jejuni*.

Cj0143c was identified as a glycoprotein modified by the Pgl system (19) and was identified in this study as having a single glycosylation site. However, glycosylation of Cj0143c is not necessary for the function of the protein in the assay results examined, including chick cecum colonization. The level of glycosylation remained unchanged under different conditions, including temperature, zinc content, growth phase, and atmosphere (data not shown). The N glycosylation system is necessary for a number of phenotypes, including colonization of both chicks and mice (41). Although these studies demonstrate the importance of the N glycosylation system in *C. jejuni* pathogenesis, the precise function is still under investigation. The glycosylation consensus sequence has been identified in several proteins, including Cj1496c and Cj0143c, as determined from proteomic analysis (22, 40). Both Cj1496c and Cj0143c are required for the colonization of chicks, but the glycosylation of both proteins is not (this study; 20). Conversely, the glycosylation of VirB10, a putative structural component of the pVir type IV secretion system, was demonstrated to be necessary for competence (23). The exact mechanism of the N glycosylation system and why it is necessary in chicks and why the Cj1496c and Cj0143c proteins are glycosylated are still unclear.

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